2017

DNA Binding Modes of Novel Terpyridine Ruthenium(II) Complexes Containing a Chloroquine Analogue

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DNA BINDING MODES OF NOVEL TERPYRIDINE RUTHENIUM(II) COMPLEXES CONTAINING A CHLOROQUINE ANALOGUE

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Honors thesis submitted to the University of Redlands Department of Chemistry Faculty in partial fulfillment of the requirements for the degree of

BACHELOR OF SCIENCE
CHEMISTRY

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March 2017
Redlands, California
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ACKNOWLEDGEMENTS

I would like to thank the University of Redlands Department of Chemistry faculty and staff for their unmatched dedication to chemistry and their students, for the opportunity of conducting research, and for the academic and cordial community that not many will experience. I extend my gratitude to my peers who have accompanied me on this journey with their camaraderie: Aaron Jensen, Jacob Khouri, Sofia Patterson, among others.

Of the faculty, I would like to first acknowledge my research advisor, Dr. Acquaye, for sharing shards of his expertise in inorganic chemistry and his guidance throughout this project. Secondly, my academic advisor, Dr. Van Engelen, for inspiring the momentum that allowed me to plunge myself into Chemistry. Without her resonating passion in those first crucial semesters of General Chemistry, I would not have chosen this path.

I would also like to thank my Honors committee for their service and advice, especially Dr. Wacks who has unveiled the world of biochemistry in a convention unique to his personality, teaching style, and absolutely pure commitment to academia.

Lastly, I would like to acknowledge the great debt I owe to my family for their continued support, love, and understanding.
The discovery of *cis*-platin as a chemotherapeutic agent has pioneered the search for superior anticancer drugs within the domain of inorganic chemistry. Ruthenium complexes have had quite the spotlight in this search due to their chemical and biological properties; NAMI-A and KP1019, two ruthenium anticancer drugs, have made it to clinical trials. Ligands of the polypyridyl class are known to be good intercalators. Chloroquine, aside from its antimalarial qualities, has induced apoptosis in several tumor cell lines of glioma and when combined with ruthenium, has exhibited the induction of apoptosis upon multiple cell lines. The synthesis, spectroscopic characterization, and DNA binding interactions have been studied for novel ruthenium complexes of the general formula: \([\text{Ru}(x\text{-tpy})(Y)(L)]\), where \(x\) is the modification of the terpyridine ligand (\(x=\)hydrogen, phenyl, tolyl, methoxyphenyl), \(Y\) is either a -chloro or -aquo ligand, and \(L\) is the chloroquine analogue ligand. Absorption titration and competitive binding studies with ethidium bromide have produced binding constants within the magnitude of \(10^5\) and \(10^4\), respectively, suggesting that the complexes bind fairly strongly to CT-DNA. Thermal denaturation studies indicate that our complexes do intercalate with DNA, while other possible DNA binding modes inherent to our complexes (phosphodiester cleavage, minor groove binding, adduct formation) have not been determined with precision.
GLOSSARY

Many abbreviations are used in this report. The following figures are accompanied with a caption (below) in the order of: **abbreviation**, formal name (not chemical name), and their description, “purpose”, or role in our study.

**L1P** - Ligand 1 Precursor. *Used to synthesize the chloroquine analog ligand, L1.*

**L1** - Ligand 1. *The chloroquine analog that will appear in all complexes used for DNA studies.*

**CxP** - Complex x Precursor. *This is the general abbreviation for all complex precursors, where x is the derivatization of the terpyridine ligand. For example, “Synthesis of CxP” would indicate that the following experimental procedure refers to the synthesis of complex 1 precursor, complex 2 precursor, complex 3 precursor, and complex 4 precursor.*

**tpy** - terpyridine. *The terpyridine ligand associated with complexes of the number 1 (Complex 1 Precursor, Complex 1, and aquo Complex 1).*
ptpy - phenyl terpyridine. The terpyridine ligand associated with complexes of the number 2 (Complex 2 Precursor, Complex 2, and aquo Complex 2).

ttpy - tolyl terpyridine. The terpyridine ligand associated with complexes of the number 3 (Complex 3 Precursor, Complex 3, and aquo Complex 3).

mtpy - methoxy phenyl terpyridine. The terpyridine ligand associated with complexes of the number 4 (Complex 4 Precursor, Complex 4, and aquo Complex 4).

C1P - Complex 1 Precursor. This complex is used to form the final ruthenium complex that will feature the chloroquine analog. A terpyridine ligand (shown by the N-N-N) and three chloro ligands are present in this particular molecule.
Cx - Complex x. General abbreviation that refers to any and all chloro ruthenium complexes. The derivatization of the terpyridine ligand and subsequent number of the chloro complex is governed by the terpyridine ligand.

C1 - Complex 1. One of four species of our chloro ruthenium complexes used for DNA studies. This complex features a terpyridine ligand (shown by the N-N-N), the chloroquine analog L1 (shown by the N-OH), and a chloro ligand.

C2 - Complex 2. One of four species of our chloro ruthenium complexes used for DNA studies. This complex features a phenyl terpyridine ligand, the chloroquine analog L1, and a chloro ligand.

C3 - Complex 3. One of four species of our chloro ruthenium complexes used for DNA studies. This complex features a tolyl terpyridine ligand, the chloroquine analog L1, and a chloro ligand.
**C4** - Complex 4. *One of four species of our chloro ruthenium complexes used for DNA studies. This complex features a methoxy phenyl terpyridine ligand, the chloroquine analog L1, and a chloro ligand.*

**aCx** - aquo Complex x. *General abbreviation that refers to any and all aquo ruthenium complexes. The derivatization of the terpyridine ligand and subsequent number of the aquo complex is governed by the terpyridine ligand.*

**aC1** - aquo Complex 1. *One of four species of our aquo ruthenium complexes used for DNA studies. This complex is similar to the chloro form, except that the chloro ligand has been replaced by an aquo ligand.*

**aC2, aC3, aC4** - aquo Complexes 2, 3, and 4. *Similar in structure to their chloro forms, except all chloro ligands have been replaced by aquo ligands.*
INTRODUCTION

Due to the increasing amount of lives affected by cancer, scientists have been searching for cures through multiple ways, such as synthesizing and testing prospective drugs that have exhibited potential for ceasing cell growth. One such drug is the complex cis-platin which was observed to stop cell division of the bacterium *Escherichia coli*[^1^], and ultimately showed promising inhibition of tumor cells in mice[^2^]. This finding in the mid-late 1900s pioneered the way for platinum-based anticancer chemotherapeutic agents. Beginning with the development of this well known agent *cis*-platin, anticancer research within the field of inorganic chemistry has expanded in the search for alternative chemotherapeutic drugs.

Despite the effectiveness and sheer power of *cis*-platin, it certainly has its disadvantages. *Cis*-platin leads to multiple problematic side effects, such as nausea, nerve damage, and anemia[^3^], which are all theorized to be attributed to the transition metal platinum. These side effects could be a response to abnormally large levels of platinum and may be ways to expel the chemical out of the body. Consequently, researchers have been studying other transition metals such as ruthenium, copper, and vanadium, to synthesize less toxic and more efficient drugs. Ruthenium is of particular interest since it has already played a key role in multiple anticancer agents currently advancing in clinical trials[^4^]. This is due in part to the favorable properties of ruthenium. These properties have granted the transition metal increased effectiveness, ligand versatility, and lower toxicity[^5^].

The effectiveness of ruthenium is attributed to the mechanism by which it is introduced to a cancer cell[^5^]. It is thought that ruthenium has the ability to mimic iron and can bind to commonly found proteins such as albumin and transferrin[^4^], decreasing the likelihood that
ruthenium will be rejected by the body as an alien species. These two proteins are used to transport iron and thus will be able to transport ruthenium. The ability of ruthenium to bind to transferrin was observed in a study conducted by Kratz et. al\cite{6}. They found that the anticancer ruthenium agent KP1019, which is undergoing clinical trials, was able to bind to transferrin and retain its ligands; iron complexes usually lose their ligands when loading onto transferrin.

Because rapidly dividing cells, such as cancer, have a greater demand for iron among other nutrients, proteins that have bound to ruthenium are able to deliver the metal to the cancer cells at a greater frequency\cite{7}. It has been observed that because ruthenium complexes often undergo active transport or passive diffusion into a cell, cancer is less likely to develop a resistance toward the drug\cite{5}, bolstering the ruthenium complexes’ effectiveness.

There are multiple membrane proteins that facilitate the transport of molecules across a cell membrane, and one such class of proteins is the ATP-binding cassette (ABC) superfamily. ABC proteins provide one of the most significant forms of resistance to drugs by expelling cytotoxic agents, ultimately keeping intracellular concentrations below a cell-killing threshold\cite{8}. Some ABC proteins are encoded in the multidrug resistance gene (MDR) which is often overexpressed in some tumors; such as lung and colon carcinomas and hepatomas; making the transport of anticancer agents across the cell membrane less likely. In tandem with the ability of ruthenium to bind to transferrin and cancer cells demand for iron, transferrin-bound ruthenium anticancer drugs may be able to bypass the resistance posed by the MDR gene and ABC proteins by endocytosis, the mechanism that iron-loaded transferrin enters cells.

Ruthenium has a substantially large amount of chances to form a complex with anticancer properties due to the amount of combinations we can achieve with the variety of
oxidation states and six-coordinate nature, a property known as ligand versatility. For example, a chloro-ruthenium complex that loses its chloro ligand and acquires an overall positive charge, should not decompose due to stability issues. Additionally, ruthenium is a six-coordinate metal with octahedral geometry and provides much opportunity for organizing many ligand species in three dimensional space, as opposed to *cis*-platin whose ligands are in two dimensional space. Comparing the possible ways $[\text{Ru(bipy)}_3]^{2+}$ and square-planar complexes can intercalate with DNA, $[\text{Ru(bipy)}_3]^{2+}$ has three planes of intercalation as opposed to the one plane of a simple square-planar complex. Overall, the ruthenium complex is more likely to intercalate when approaching DNA because $[\text{Ru(bipy)}_3]^{2+}$ has a higher chance of being in the right orientation for interaction.

The effect of ligand versatility can be observed in ruthenium arene complexes, also known as “half-sandwich” complexes, which have displayed increased anticancer activity and strong binding interactions with DNA -- not all transition metals can form hexahapto-arene complexes. Lower toxicity is attributed to the variety of oxidation states and its ability to be compatible with biological fluids.

When a ruthenium complex penetrates cancer cells, it is able to interact with the cancer cell’s DNA, distorting or maiming the strands and eventually causing apoptosis, or programmed cell death. Although the DNA is wrapped around histones, enough of the DNA is exposed for cytotoxic molecules, for example, to interact with it. These histones are modified and released from the DNA strands as apoptosis occurs. There are multiple factors that can determine how well a complex can inhibit cancer cell growth through DNA interaction: (1) size/shape, (2) intermolecular and covalent interaction, and (3) composition.
Firstly, small molecules are able to interact with DNA through several non-covalent modes: groove binding, intercalation, and external static electronic effects\textsuperscript{11}. In some cases, anticancer drugs may prefer to intercalate with DNA by inserting between two DNA base pairs. Because of the minimal amount of space in between each DNA base pair, it would be expected that bulkier, multidimensional ligands would have a decreased activity of intercalation compared to ligands of lower dimensions, i.e. planar molecules. In fact, two ruthenium complexes that have entered clinical trials as potential anticancer agents: NAMI-A and KP1019\textsuperscript{12}, feature planar ligands. Studies have shown that such molecular qualities are more effective at causing apoptosis, and even some molecular classes --like polypyridyl ligands--have shown remarkable promise in DNA intercalation\textsuperscript{4}. In the recent years, bipyridyl and terpyridyl complexes have been studied alongside a series of other polypyridyl ligands such as; phenanthroline, dipyridophenazine (dppz), etc. Aside from the common planar quality of all the aforementioned ligands, they all have other electroactive subunits that will alter the complexes’ ability to bind to DNA\textsuperscript{13}. Particularly, ruthenium complexes containing terpyridine have been found to covalently bind to DNA to form monofunctional adducts, while some of these complexes were able to stop DNA replication\textsuperscript{14}.

Secondly, the degree of DNA binding interaction can be examined through a complexes’ ability to covalently interact with the electron-rich DNA bases or phosphate groups\textsuperscript{13}. The phosphodiester backbone of DNA is able to be cleaved by nucleophilic attack\textsuperscript{15}, and if an incoming complex features a nucleophilic site, the complex is able to cleave the DNA strand to break phosphodiester bonds. Ultimately, the replication and transcription of DNA is prevented. In theory, an efficient anticancer drug would be able to both intercalate and cleave DNA, but the
experimental strength of the anticancer drug can only be compared with and measured by numerical values. Once prospects from anticancer drug research have been unveiled, these drugs may then be studied for their cytotoxicity in vitro\textsuperscript{16}.

Thirdly, complexes that contain halide leaving groups are able to react readily once they have traversed through the cell membrane\textsuperscript{17}. In a case study of \textit{cis}-platin, the chloro ligands of the complex are displaced by aquo ligands, allowing the aquo species to disturb the DNA structure. This can be explained by the chloride concentrations between blood and cytoplasm which differ by about a factor of ten, with the concentration of chloride anion being lower in cytoplasm\textsuperscript{18}. Due to the common ion effect, an anticancer drug containing chlorine will be less likely to dissociate in blood than in cytoplasm. Once the anticancer drug has entered the cell and resides in the cytoplasm, the chloride ligand is readily replaced with a water ligand in a process called aquation. Now that there is an aqua ligand on the anticancer complex, the aqua ligand is then displaced and substituted by an \textit{N}-heterocyclic base of DNA, preferentially by the guanine unit\textsuperscript{18}, resulting in a DNA-complex form. Because the DNA subunit is bound to the complex, the cell recognizes that the DNA must be repaired, but eventually activates apoptosis since DNA repair is impossible.
Certain polypyridyl ruthenium complexes have been studied in the past for their interactions with DNA. Cytotoxicity was observed in many Ru(II) and Ru(III) complexes, including: *mer*-[^TPGCI]Ru(tpy)Cl$_3$[^19], *α*-[^TPGCI]Ru(azpy)$_2$Cl$_2$[^20], and hexahapto arene-Ru complexes[^21]. Reactivity with CT-DNA has been observed in a wide variety of complexes too, such as; [Ru(phen)$_2$(dppz)][^22] and [Ru(tpy)(HPB)(H$_2$O)][^23] where HPB is 2-(2’-hydroxyphenyl)-benzoxazole. Additionally, certain ruthenium complexes are especially photoreactive and have efficient DNA photocleavage abilities[^24][^11], such as [Ru(bpy)$_3$]$^{2+}$ where bpy is 2,2’-bipyridine[^25].
In the effort to develop an anticancer drug better than those currently available, scientists have been incorporating other substances in their studies. One such substance is chloroquine, well known for its antimalarial activity. Chloroquine fights malaria-infected cells by inducing or provoking cell death by apoptosis. The degree of antimalarial activity among a variety of chloroquine analogues was studied, and it was determined that the most efficient antimalarial chloroquine analogue contained a salicylaldiminate unit\cite{26}. Additionally, chloroquine has also been found to induce apoptosis in several tumor cell lines of glioma\cite{27}, suggesting that the chloroquine class of compounds can combat not only malaria, but also forms of cancer. Through multiple studies, it has been determined that chloroquine by itself is already an effective anticancer agent, induces preventive effects, and enhances the effectiveness of other anticancer drugs when used in tandem\cite{28}. Furthermore, researchers found that chloroquine is relatively concentrated in multiple parts of the body\cite{7}. In animal studies, it was found that, in comparison to plasma; chloroquine is hundreds of times more likely to be found in the liver, spleen, kidney,
and lungs; dozens of times more likely to be found in the brain and spine; and since chloroquine has been observed to strongly bind to melanin, chloroquine can also be found in the eyes and skin in more concentrated amounts\cite{7}. Therefore, if an anticancer drug is able to incorporate chloroquine in its design, that drug may be able to treat a large variety of cancers.

\begin{center}
\includegraphics{chloroquine.png}
\end{center}

\textbf{Figure 3. Chloroquine.}

In another study, the DNA binding interactions of arene ruthenium complexes of chloroquine were studied\cite{27}. It was discovered that the complexes induced cytotoxicity through apoptosis\cite{9}. This was due to an observed strong binding interaction between the cancer cell’s DNA and the chloroquine ligand\cite{9}. The chloroquine ligand is able to intercalate between the DNA base pairs, and the terminal side of chloroquine displayed strong electrostatic interactions with the phosphate groups of the DNA. It is anticipated that with these conclusions about the DNA binding interactions of chloroquine, a similar effect will take place when the ruthenium complexes and modified chloroquine ligands are tested with CT-DNA, or calf-thymus DNA. Hence, it is expected that the chloroquine ligand will have a stronger DNA binding interaction than the polypyridyl ligand and will be more likely to intercalate with DNA. Since the DNA-binding properties of the entire complex can be attributed to the ancillary ligand, or the ligand that is not directly involved in the DNA-complex interaction, it is expected that the degree of anticancer activity will increase as the plane of the ancillary ligand is extended\cite{13}. For example, a complex containing chloroquine and 4’-phenyl-2,2’:6’,2”-terpyridine
(phenylterpyridine) should display a stronger DNA binding interaction than a complex containing terpyridine. In contrast, if the ancillary ligand contains any sort of substituent that could potentially form a covalent bond with the phosphodiester components of the DNA, then perhaps the role of “ancillary ligand” will switch from the modified terpyridine to the chloroquine (in this particular case).

In order to determine the binding strength of our complexes, multiple experiments were employed: absorption titration, competitive binding with ethidium bromide, thermal denaturation, and DNA cleavage.

Absorption Titration is both a qualitative and quantitative method for determining the binding strength of a complex to a DNA substrate. Quantitatively, the binding strength can be determined through the intrinsic binding constant. Favorable intercalative intermolecular forces (hydrophobic, ionic, hydrogen bonds, van der Waals) contribute to binding constants of $10^5$ to $10^{11} \text{ M}^{-1}$ [29]. Particularly, two ruthenium anticancer complexes that have made it to clinical trials (NAMI-A and KP1019) were observed to have $K_b$ values of $10^7 \text{ M}^{-1}$.

The qualitative properties of the absorption titration experiment lay within the absorbance spectra collected. Addition of CT-DNA substrate to the complex can result in hypochromism, hyperchromism, and bathochromic shift. Hypochromism (decrease in absorbance) indicates a shift to lower energy meaning the DNA-complex form is a favorable interaction. Hyperchromism indicates a breakage of DNA secondary structure by complex coordination to the phosphate backbone or simply indicates covalent binding. Bathochromic shift indicates a coordination between the complex and the N7 of guanine [29] and intercalation association between the complex and CT-DNA [30].
Competitive Binding with Ethidium Bromide is another DNA-binding experiment that is widely used to qualitatively and quantitatively measure the binding strength of a complex to DNA by determining the stern-volmer constant, $K_{sv}$. Ethidium bromide is a known carcinogen that can easily slip through human skin cells and intercalate with DNA. Ethidium Bromide has an intrinsic binding constant of $7.16 \times 10^5\text{ M}^{-1}$\[^{29}\]. Competitive binding assays are unique to our cause in that they serve as a qualitative indicator of intercalation. Ethidium bromide is a fluorescent molecule and as it intercalates with a DNA substrate, the intensity of the fluorescence increases. If the intensity decreases as a complex is introduced to this system, the assumption that the complex is intercalating and binds to DNA more strongly than the bond made by ethidium bromide\[^{31}\]. This assumption is based entirely around the fact that the fluorescence intensity relies upon ethidium bromide and DNA intercalation. The linearity of the plot(s) obtained from the competitive binding experiments can indicate whether a complex is breaking down. If the plot is linear, then only one quenching process is occurring within the system. If a non-linear graph is obtained, then the complex introduced may be either impure or degrading to produce two or more intercalators (that can displace ethidium bromide).

In addition to ethidium bromide, other reagents such as Hoechst-33342, shown in Figure 4, have been used in the past to determine the preferential DNA interaction of a complex or compound. Particularly, Hoechst-33342 forms a DNA-Hoechst-33342 complex via a minor groove bond\[^{33}\]. With data collected from competitive binding experiments with these two binding agents, Du et. al. discovered that one of their complexes favored minor groove binding over intercalation. Although the chloroquine analog ligand isn’t quite as long as Hoechst-33342,
it could be possible that the bidentate ligand and its dangling 7-chloro-4-quinolyl end could bind to DNA in a minor groove mode.

![Chemical structure](image)

**Figure 4.** The chloroquine analog ligand research in our research (top) and Hoechst-33342 (bottom).

In thermal denaturation, the melting temperature of DNA increases when metal complexes bind to DNA by intercalation[^11]. Intercalation causes the DNA base pairs to stabilize via base stacking and so the melting temperature of the double-stranded DNA will increase. Thermal denaturation of DNA with a classical intercalator (ethidium bromide) and several ruthenium polypyridyl complexes were studied[^34]. With ethidium bromide as an indicator for an increase in melting temperature (+13 °C), they found the increase in melting temperature for 

\[
[Ru(tpy)(bpy)OH_2]^{2+} (+4.2 \pm 0.5 \, ^\circ \text{C}), [Ru(tpy)(phen)OH_2]^{2+} (+7.2 \pm 0.4 \, ^\circ \text{C}), \text{ and} \\
[Ru(tpy)(dppz)OH_2]^{3+} (+14.1 \pm 0.8 \, ^\circ \text{C}).
\]

DNA cleavage via hydrolytic cleavage of supercoiled plasmid DNA by a ruthenium polypyridyl complex was studied by Deshpande et. al.[^35]. Cleaving agents were more successful
if they induced hydrolytic cleavage as opposed to oxidative cleavage. Oxidative cleaving agents ultimately result in DNA fragments that cannot be re-ligated, whereas hydrolytic cleaving agents do not suffer from this drawback\textsuperscript{[35]}. They found that a ligand which featured a urea moiety, specifically a bipyridyl glycouril ligand, was sufficiently able to facilitate the hydrolysis of the phosphate backbone. Upon cleavage of the phosphate backbone, the supercoiled plasmid DNA is converted into a nicked circular form. This change in DNA structure can be monitored through gel electrophoresis. If a present complex is able to nick the DNA, the nicked form will travel through the gel slower than the supercoiled form, as the structure of the nicked form is essentially larger in volume. With an ethidium bromide stain, the DNA sample can be viewed under UV light and the degree of hydrolytic activity can be determined qualitatively.

Herein, we report the synthesis of ruthenium complexes that feature qualities of a theoretically effective intercalating and hydrolytic cleaving anticancer agent. We have derived the synthesis of a new chloroquine analogue from a similar scheme by Natarajan et al.\textsuperscript{[36]} and coordinated a modified terpyridine ligand to a ruthenium center. The complexes’ binding interactions with calf thymus DNA (CT-DNA) has been studied with a series of Ru(L)(X-tpy)(Y) complexes; L is the chloroquine analog ligand, xtpy is the modified terpyridine ligand (x = H, phenyl, tolyl, methoxyphenyl), and Y is the leaving group (-Cl or -H\textsubscript{2}O). All complexes formed have been designed to have at least one chloride leaving group. Subsequently, the corresponding aquo form of each complex have been synthesized. Several methods utilized to study these interactions include: absorption titration, competitive binding with ethidium bromide, thermal denaturation, and DNA cleavage. The intrinsic binding constants for some chloro and aquo complexes have been determined (magnitude of $10^5$ M\textsuperscript{-1}), as other complexes
did not indicate a favorable interaction with CT-DNA. Stern-Volmer constants have been
determined for all complexes synthesized (magnitude of $10^4 \, \text{M}^{-1}$) indicating intercalation and the
displacement of ethidium bromide, and the plots obtained resulted in a slightly non-linear
relationship, indicating that the complex is not stable in physiological conditions. In thermal
denaturation, we will be able to observe our complexes intercalating with DNA if there is an
increase in melting point of the DNA. With DNA cleavage through gel electrophoresis, we will
be able to see whether our complex has a suitable nucleophilic site that can cleave the
phosphodiester bond of supercoiled plasmid DNA. Lastly, once all complexes have been studied,
we will be able to draw any correlations between the modification of terpyridine and the effect it
has on DNA interactions.
◆ EXPERIMENTAL METHODS

Materials

All solvents and materials used for preparation were reagent grade, purchased from Sigma Aldrich Company based in St. Louis, MO, and used as received without further purification.

Instrumentation

Absorbance spectroscopic measurements were recorded using a 1.0 cm quartz cuvette on a Shimadzu PharmaSpec 1700 UV/Vis Spectrophotometer. For thermal denaturation studies, absorbance spectra were recorded on a Hitachi U-2900 Double-beam UV-Visible Spectrophotometer accompanied by a Lauda Eco E4G Heating Thermostat.

Liquid chromatography mass spectrometric measurements were recorded with an Agilent 6530 HPLC-QTOF High Performance Liquid Chromatography-Tandem Mass Spectrometer.

Nuclear magnetic resonance spectroscopic measurements were recorded with a Varian 400-MR 400 MHz Nuclear Magnetic Resonance Spectrometer. All samples were dissolved in deuterated DMSO.

Cyclic Voltammetry studies were performed in dichloromethane with a BASi Epsilon EClipse Electrochemical Analyzer. The single compartment cell was equipped with three electrodes: a platinum disk working electrode, a platinum wire auxiliary electrode, and a Ag reference electrode in saturated KCl solution. A supporting electrolyte, 100 mg of tetrabutylammonium hexafluorophosphate (Bu$_4$NPF$_6$) was employed. Scans were taken at 100 mV/s with a 10 uA full scale current.
Syntheses:

I. Synthesis of Main Ligand Precursor (L1P)

4,7-dichloroquinoline (2.000 g, 10 mmol) and excess ethylenediamine (3.4 mL, 50.9 mmol) were combined in a round bottom flask. The mixture was heated and stirred for six hours at 100°C under inert conditions. 20 mL 1 M NaOH was added slowly and swirled to precipitate a light-tan powder. The powder was vacuum filtered, washed with dichloromethane and deionized water, then dried overnight under reduced pressure. Yield: 90.5%

II. Synthesis of Main Ligand (L1)

L1P (443.5 mg, 2.00 mmol) and 2-hydroxy-1-naphthaldehyde (344.3 mg, 2.00 mmol) were combined in a round bottom flask. The mixture was refluxed and stirred in ethanol for 14 hours at 100°C under inert conditions. After the reaction was completed, the reaction mixture was reduced to about 50% of the initial volume by rotary evaporation. Aliquots of water were added to the reduced reaction mixture to precipitate a yellow powder. The mixture was vacuum filtered to complete dryness for several hours. Yield 88.65%
III. **Synthesis of 4’-(4-phenyl)-2,2’:6’,2”-terpyridine (ptpy)**

60.0 mL of 1.5 M NaOH and 40.0 mL of ethanol were added to a round bottom flask and stirred with a magnetic stir bar. While stirring, benzaldehyde (4.600 mL, 45.08 mmol) was drawn into a 1.000 volumetric pipet and slowly added to the round bottom flask. 2-acetylpyridine (10 mL, 89.2 mmol) was added dropwise to the round bottom flask. The contents were stirred overnight to produce a slightly pink, milky mixture. The precipitate--referred to as “dione”--was vacuum filtered, washed with ethanol, and dried for about 30 minutes. Yield 87.2%

100 mL of ethanol was added to a round bottom flask. Ammonium acetate (3.084 g, 40.0 mmol) was added to the flask at which point the solution was heated to a boil and stirred. The dry dione (6.0261 g, 18.3 mmol) was added to the boiling solution scoop wise, and the reaction was refluxed for one hour. A microcrystalline yellow precipitate was isolated via vacuum filtration where it was washed with ethanol until the immediate filtrate was clear. The product was stored in a glass vial wrapped in foil to protect it from light. Yield 66.9%
IV. Synthesis of Ruthenium Complex Precursor (CxP*)

RuCl₃•H₂O (530 mg, 2.56 mmol) and 50 mL of 95% ethanol were added to a round bottom flask. The solution was degassed with nitrogen for at least three minutes. 4’-(4-X)-2,2’:6’,2’’-terpyridine, where “X” = hydrogen, phenyl, tolyl, or methoxyphenyl, (C1P: 600 mg, 2.57 mmol; C2P: 791 mg, 2.56 mmol; C3P: 826 mg, 2.56 mmol; C4P: 867 mg, 2.56 mmol) was added to a round bottom flask. The mixture was refluxed in ethanol and stirred overnight under inert conditions. The volume of the solution was reduced to about 50% of the initial volume by rotary evaporation. The resulting contents were then vacuum filtered to afford a dark brown powder (C1P, C2P) or a dark purple/brown powder (C3P, C4P). The precipitate was washed with chilled ethanol until the immediate filtrate was clear. Yield C1P: 96.5%, C2P: 52.2%, C3P: 84.4%, C4P: 76.5%
*"x" represents the placeholder for the species number of ruthenium complex, e.g. C1P denotes Complex 1 Precursor, C2 denotes chloro-Complex 2.

**Figure 8. Synthesis of CxP.**

V. **Synthesis of chloro-Ruthenium Complex (Cx)**

Ruthenium precursor, CxP, (200 mg, C1: 0.454 mmol, C2, C3, C4: 0.500 mmol), main ligand, L1, (185.3 mg, 0.493 mmol) and excess LiCl were added to a round bottom flask. The contents were dissolved in 75 mL ethanol, 25 mL deionized water, and 1 mL triethylamine. The solution was degassed with nitrogen for at least three minutes then refluxed and stirred for four hours in inert conditions.

The contents of the round bottom flask were then dried to complete dryness via heated rotary evaporation. 20 mL ice-cold water was added to loosen the solid contents. The mixture was then vacuum filtered to complete dryness overnight. Yield C1: 95.2%, C3: 99.0%, C4: 97.0%

Specifically for C2, the dried precipitate was washed with water several times to yield an oily dark-red solution. The solid precipitate was redissolved in ethanol and vacuum filtered. The filtrate was passed through an alumina column with a dichloromethane mobile phase to separate
an oily dark-red elute. Later, a methanol mobile phase was used to elute the purple-black product. The methanol phase was allowed to evaporate over a few days to isolate the purple-black product. Yield C2: 51.4%

![Complex X Precursor, Main Ligand 1, Chloro-Complex X](image)

**Figure 9.** Synthesis of Cx.

**VI. Synthesis of aquo-Ruthenium Complex (aCx)**

The chloro-form, aCx, (200 mg, 0.268-.235 mmol depending on the species) was added to a round bottom flask and dissolved in 50 mL water and 20 mL of acetone. The solution was degassed with nitrogen for at least three minutes. A small excess of AgPF$_6$ (~100 mg, 0.690 mmol) was added to the reaction mixture and was refluxed for two hours under inert atmosphere. The gray AgCl precipitate was vacuum filtered through a sintered glass filter and the precipitate was washed with acetone to transfer all ruthenium product into the filtrate. Excess NH$_4$PF$_6$ was then added to the filtrate and the solution was stored in the refrigerator for at least 2 days.

The refrigerated filtrate was vacuum filtered to collect the aquo-ruthenium complex form that had precipitated. Dark-purple microcrystalline structures were afforded. The final filtrate from this second filtration was allowed to sit in a dark, dry place to allow more aquo-form to precipitate. Yield aC1: 69.8%, aC2: , aC3: 79.7%, aC4: 50.3%
DNA Reactivity Studies:

Preparation of the Phosphate Buffer

1.2 g (10.0 mmol) of Na$_2$PO$_4$ was dissolved in about 900 mL of deionized water. 7.577 g of NaCl was added to the solution. The solution was then titrated with 0.02 M NaOH to achieve a final pH of 7.2 and the solution was diluted to 1000 mL, resulting in a 0.01 M PO$_4^{3-}$ buffer (pH=7.2) prepared at 25 °C to be used at 37 °C.

Preparation of the Ethidium Bromide Solution

A 3.7 mg (9.4 μmol) sample of ethidium bromide was dissolved in 25 mL of phosphate buffer and stored in a glass bottle.

Preparation of the CT-DNA Solution

Deoxyribonucleic acid from activated calf thymus, or CT-DNA, was purchased from Sigma Aldrich. The solid CT-DNA was funneled into a 10.00 mL volumetric flask using phosphate buffer. Before the solution reached the 10.00 mL mark, the remaining solid CT-DNA
was dissolved in an ultrasonic bath. Once all the CT-DNA was dissolved, the solution was brought to the mark and this “stock” solution was transferred into a small amber glass bottle. A 3:8 dilution was performed unto the stock CT-DNA solution to produce appropriately concentrated solutions of CT-DNA. The concentration of the CT-DNA was determined by UV-Vis spectroscopy. With an extinction coefficient of 6600 M$^{-1}$cm$^{-1}$ the concentration of the diluted DNA solution was determined based on the absorbance value at 260 nm. This diluted DNA solution was used for all CT-DNA studies.

**Preparation of Complex Solution for Absorption Titration and Competitive Binding DNA Studies**

A small sample (5.0-7.0 mg, 8.7-6.6 μmol) of solid complex was dissolved in 10.00 mL methanol and stored in a glass bottle.

**VII. Absorption Titration**

All spectroscopic analyses were performed on a Shimadzu PharmaSpec 1700 UV/Vis Spectrophotometer. The absorption titration experiments were performed with constant complex concentrations (25 μM) with varying CT-DNA concentrations (0-15 μM) of CT-DNA via addition of aliquots of CT-DNA solution to the spectroscopic cuvette.

A methanol blank was used to auto-zero and baseline the spectrophotometer. An appropriate amount of methanol was removed and replaced with complex solution. A 10 μL aliquot of CT-DNA solution was added to the same cuvette and the solution was allowed to incubate at 37 °C for at least 10 minutes. Spectra were recorded between each aliquot addition.
10 µL aliquots were added to the same cuvette until a total of 100 µL CT-DNA has been added. The solution was never remade as the experiment progressed.

For chloro-complexes, spectra were recorded over 750-250 nm and for aquo-complexes, spectra were recorded over 950-250 nm. All solutions for the absorption titration experiment were prepared within the cuvette using automatic pipettors to produce a volume of 3.000 mL.

VIII. Competitive Binding with Ethidium Bromide

All spectroscopic analyses were performed on a JASCO FP-750 Spectrofluorometer. The competitive binding experiments were performed with constant CT-DNA concentrations (3 µM) and ethidium bromide concentrations (20 µM) and varying complex concentrations (0-20 µM). All samples were subjected to an excitation wavelength of 520 nm and emissions were recorded over 535-700 nm.

A phosphate buffer blank of 3.000 mL was used to auto-zero the spectrofluorometer. 150 µL of the buffer was removed from the cuvette and 150 µL of ethidium bromide was added. A spectrum was recorded. A new sample was prepared with 150 µL ethidium bromide solution, 20 µL CT-DNA solution, and the sample was brought to 3.000 mL. A spectrum of the reaction mixture was recorded. Separate samples were prepared with varying concentrations of complex solution (0-100 µL), 150 µL ethidium bromide solution, and 20 µL CT-DNA solution. All samples were allowed to incubate for at least 15 minutes in a 37 °C water bath. Spectra were recorded for each sample and a decreasing intensity was observed.
Preparation of Complex Solution for Thermal Denaturation

A very small sample of solid complex (3.0-3.5 mg) was dissolved in 1.000 mL DMF. Solutions were sonicated until fully dissolved. 1.000 mL of phosphate buffer was added to the solution, producing a final concentration of complex around 2.1 mM.

IX. Thermal Denaturation

All spectroscopic analyses were performed on a Hitachi U-2900 Double-beam UV-Visible Spectrophotometer accompanied by a Lauda Eco E4G Heating Thermostat. There was no “communication” between the two, meaning that the temperature could not be monitored/altered with the spectrophotometer controls. Instead of electrically heated padding, water from the thermostat’s water bath was transported via plastic tubing to heating pads directly adjacent to the cells. Because heat is lost via plastic tube transportation, the temperature of the water bath \( T_{\text{bath}} \) differed greatly from the temperature of the cell \( T_{\text{cell}} \). In order to best estimate the actual temperature within the cuvette, a “calibration phase” of the experiment was run to provide a change in temperature over time.

For the calibration phase, phosphate buffer was placed into both the sample and reference cuvettes. The reference cuvette was capped tightly while a digital thermometer was placed into the sample cuvette and remained in one single position until the end of the calibration phase. In order to constantly record the temperature, the hood of the spectrophotometer was kept open. The \( T_{\text{bath}} \) was increased by increments of 5 °C from 50 °C to 70 °C and increments of 3 °C from 70 °C to 97 °C. After each incremental increase of temperature, \( T_{\text{cell}} \) was allowed to equilibrate.
until no change in temperature was observed. The $T_{\text{cell}}$ for each corresponding $T_{\text{bath}}$ and the time required for $T_{\text{cell}}$ to meet equilibrium for each benchmark was recorded.

For the experimental phase, cells were filled with solution as completely as possible and capped tightly, this amounted to 3.500 mL. The absorbance of all solutions was recorded at 260 nm over time. The instrument was zeroed and contained a reference with phosphate buffer. The sample cuvette contained 200 µL (35.9 µM) DNA, 50 µL (30 µM) complex, and 3.250 mL phosphate buffer.

The absorbance at 260 nm was monitored over an estimated $T_{\text{cell}}$ range of 51.2 to 79.4 °C which corresponded to a $T_{\text{bath}}$ range of 79.0 - 97.0 °C. To determine that the $T_{\text{cell}}$ had reached equilibrium at each benchmark without dipping a digital thermometer into the sample cuvette, the time required for $T_{\text{cell}}$ to reach equilibrium in the calibration phase for each benchmark was allowed to pass. For example, if 20 minutes was required for $T_{\text{cell}}$ to reach equilibrium when increasing $T_{\text{bath}}$ from 79.0 to 82.0 °C in the calibration phase, then a minimum of 20 minutes was allowed to pass during the experimental phase when increasing $T_{\text{bath}}$ from 79.0 to 82.0 °C. The absorbance at each benchmark was recorded.

It should be noted that the change in melting temperature is directly proportional to the ratio of concentrations of intercalator to DNA, i.e. the greater amount of intercalators present for a fixed amount of DNA, more base-stacking occurs, the greater the change in melting temperature. It is for this reason the concentration of complex in the sample cell was kept relatively consistent across all experiments.
Preparation of Complex Solution for DNA Cleavage

The solutions for DNA cleavage were made from the solutions for thermal denaturation. The thermal denaturation solutions were diluted by a factor of 2, maintaining the composition of DMF:phosphate buffer. As a result, a very small sample of solid complex (3.0-3.5 mg) was dissolved in a total of 2.000 mL DMF and 2.000 mL phosphate buffer.

Preparation of pUC18 plasmid DNA Solution for DNA Cleavage

A sample of pUC18 plasmid DNA (14 µL, 10 µg) was obtained in a small plastic container. The contents of the plastic container were diluted with aliquots of phosphate buffer and the resulting solution was transferred into a plastic microfuge tube. The plastic container was washed with several more aliquots to extract all plasmid DNA solution. The final volume of the diluted plasmid DNA stock solution was 0.333 mL.

X. DNA Cleavage with Gel Electrophoresis

The gel was prepared 1x TBE buffer (125 mL) and Agarose, LE (1.25 g) in a Erlenmeyer flask. The mixture was microwaved for 75 s, and microwaved in pulses of 5-10 s until the agarose was fully dissolved. A sample of a pre-prepared solution of 1% ethidium bromide (6.25 uL) was added to the agarose solution. After allowing this solution to cool down to around 70 °C to avoid ethidium bromide fumes, the solution was added to the gel electrophoresis gel cast. The comb (20 slot) was inserted and the agarose gel was allowed to solidify for, at least, an hour.

Samples for gel electrophoresis were prepared in disposable plastic microfuge tubes. The tubes contained fixed amounts of diluted plasmid DNA stock solution (15 µL), a varying amount
of the DNA Cleavage complex solution (2 µL, 10 µL, 36.5 µL), and all samples were diluted to 50.0 µL with phosphate buffer. All tubes were incubated at 37 °C for about 1 hr. 6x blue/orange loading dye (10 µL) was added to each microfuge tube and mixed with a vortex mixer.

Portions of each solution with complex (20 µL), a molecular weight marker, and a positive blank that contained only DNA and phosphate buffer, were added to their respective wells. The forms were separated over two hours under 60 V.
**Instrumental Analysis:**

**Preparation of Liquid Chromatography Mass Spectrometry Samples**

Solid samples (~2.5 mg) were dissolved in solvent, usually methanol and occasionally acetonitrile, to achieve a final concentration of about 25 µg/mL. All samples were analyzed under positive ion mode with either a methanol or acetonitrile mobile phase.

**Preparation of Cyclic Voltammetry Samples**

To the single compartment cell, 100 mg of tetrabutylammonium hexafluorophosphate was added. The vessel was filled with dichloromethane and degassed with nitrogen for at least one minute. A “blank” scan was conducted to test for any pre-existing impurities. Once no pre-existing impurities were found as a result of the voltammetric experiment, a spatula-tip of (a)Cx was dissolved in the cell’s dichloromethane and was further degassed with nitrogen for at least one minute.
**RESULTS AND DISCUSSION**

**Syntheses:**

I. **Synthesis of L1P**

The initial directions for this procedure was adapted from Natarajan et. al.\[36\] and required one to wash the resulting contents (with the 1 M NaOH) of the reaction in a separatory funnel. The main problem of this first method was that the precipitate would get caught in the valve of the separatory funnel and yields were quite low. Instead, the resulting contents were vacuum filtered and washed with water and dichloromethane to filter out unreacted materials, as L1P is only very slightly soluble in both solvent. Yield increased from about 40% to 90%.

<table>
<thead>
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<th>Mass-to-Charge (m/z)</th>
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<tbody>
<tr>
<td>179.0365</td>
</tr>
<tr>
<td>222.0794*</td>
</tr>
<tr>
<td>383.0829</td>
</tr>
</tbody>
</table>

*Table 1. Selected Mass-to-Charge ratios from LC-MS analysis of L1P run in positive ion mode. * indicates the most prominent peak. The expected mass for L1P is 221.07.*

II. **Synthesis of L1**

The synthesis of L1 was adapted from an experiment conducted by Ekengard et. al.\[Ekengard\] involving salicylaldehyde in place of 2-hydroxy-1-naphthaldehyde. The reaction ran well as expected and produced the schiff-base product common to both reactions, but yields were rather low for some trials of L1 synthesis. Before procedure alteration, yield ranged from about 16% to 45%, and often times multiple L1 syntheses had to be run before synthesizing complexes. To remedy this, the resulting contents were precipitated with the addition of water. Yield increased to >80%.
To confirm that the reaction had successfully run, the infrared spectrum of L1 was examined for (1) the absence of carbonyl absorption band and (2) the presence of only one amine (N-H) absorption. The presence of an aldehyde peak would indicate that there is a relatively large quantity of reactant still in the isolated “product”. This aldehyde peak would be sourced from 2-hydroxy-1-naphthaldehyde. Upon the conversion from L1P to L1, the terminal amine (-NH₂) reacts with the aldehyde from 2-hydroxy-1-naphthaldehyde to form an imine. Thus, only one N-H absorption should be present in the resulting spectrum. Figure 11 shows an IR spectrum for one trial of L1.

Figure 11. An FT-IR spectrum for L1.
L1 was subjected to LC-MS analysis (positive ion mode, methanol solvent) and the dominant peaks can be found in Table 2 below. Across the time of my, and my labmates’, research, we have been confronted by several distinct masses-to-charge ratio artifacts. Specifically, ratios (m/z) around 550 and 659. These peaks were attributed to a polyethylene glycol contaminant which may have been introduced during rotary evaporation.

<table>
<thead>
<tr>
<th>Mass-to-Charge (m/z)</th>
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<tbody>
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<tr>
<td>376.1228*</td>
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<tr>
<td>550.6300</td>
</tr>
</tbody>
</table>

**Table 2. Selected Mass-to-Charge ratios from LC-MS analysis of L1 run in positive ion mode. * indicates the most prominent peak. The expected mass for L1 is 375.11.**

L1 was also subjected to NMR analysis and the $^1$H, $^{13}$C, and DEPT spectra can be found below in Figures 12-14. A calculated spectrum is also shown which was produced on nmrdb.org. The two experimental and calculated spectra display some similarities, such as the two C-NR$_2$ peaks from the ethylamine bridge and a dense aromatic region, however there are obviously some impurities present. This was attributed to the crude isolation of L1 via solvent solubility changes. Judging from the DEPT spectrum also shown below, we can see the presence of an aldehyde carbon on the tertiary carbon (CH) chemical shifts, which would correspond to the aldehyde of the 2-hydroxy-1-naphthaldehyde starting material.
Figure 12. Experimental $^{13}$C NMR spectrum (top) and calculated/predicted spectrum (bottom). Solvent used: deuterated dimethyl sulfoxide.
Figure 13. Experimental $^1H$ NMR spectrum (top) and calculated/predicted spectrum (bottom). Solvent used: deuterated dimethyl sulfoxide.
III. Synthesis of 4’-(4-phenyl)-2,2’:6’,2”-terpyridine (ptpy)

Synthesizing phenylterpy ligand was fairly straightforward, but the ratio of desired product to unreacted dione was quite low. The final solid isolated from the experiment was recrystallized in ethanol several times before a clean sample was obtained. To determine the degree of purity for this ptpy solid (mixture), IR spectroscopy and LC-MS was used.

One stark difference between the dione and ptpy is the presence of two carbonyls in the former. This makes characterization via IR spectroscopy fairly easy, as one needs to monitor the % transmittance of the carbonyl absorption band around 1700 cm$^{-1}$. After three recrystallizations,
the carbonyl absorption band had essentially faded into the messy “IR footprint” within the spectrum. Figure 15 below shows the two spectra to emphasize the % transmittance differences.

Figure 15. FT-infrared spectra for the raw ptpy product obtained (top) compared to the spectrum for the thrice-recrystallized ptpy (bottom). The 1697 cm\(^{-1}\) peak present in the raw
product indicates that a lot of unreacted dione is still present in the sample. This peak is nearly eliminated after multiple recrystallizations in ethanol.

To affirm our belief that the ptpy solid was, in fact, pure, the substance was subjected to LC-MS analysis. Table 3 below details the prominent peaks found for this thrice-recrystallized ptpy. Although the degree of purity isn’t exactly in accordance with that of IR, we chose to use the ptpy anyway knowing that it would not coordinate to the ruthenium center and would be filtered out with washings of ethanol.

<table>
<thead>
<tr>
<th>Mass-to-Charge (m/z)</th>
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<tbody>
<tr>
<td>310.1365*</td>
</tr>
<tr>
<td>332.1175</td>
</tr>
<tr>
<td>641.2462</td>
</tr>
</tbody>
</table>

Table 3. Prominent peaks in the mass spectrum of the thrice-recrystallized ptpy. Molecular mass of phenylterpyridine is 309.13 and of the dione is 330.39.

IV. Synthesis of CxP

Complex precursors were characterized with UV/Vis spectroscopy and LC-MS. Figure 16 shows an overlay of UV/Vis spectra for C1P, C1, and aC1. The two high-intensity peaks in the ultraviolet range are characteristic to the absorbance of coordinated-terpyridine and affirm that the terpyridine has coordinated to our ruthenium center. The overlay spectra for other complex species can be found in the appendix.
Figure 16. The UV/Vis spectra for C1P, C1, and aC1. Note the presence of the 740 nm peak unique to the aquo-form.

Table 4 shows the dominant peaks in the liquid chromatography mass spectrum for all CxP (positive ion mode, methanol solvent). The expected mass-to-charge ratio was 439.91 m/z and, what we claim to be, the found ratio responsible for C1P was 404.9288. The difference between these two peaks was roughly 35 m/z which corresponds to a chlorine. The hypothesis that one of three chloro ligands were dissociated from the Ru(tpy)Cl\(_3\) complex. However, there were no counts for \(^{35}\text{Cl}\) or \(^{37}\text{Cl}\) and these chloro ligands may have coordinated (or reacted) with something else within the instrument. The trend for one chloro ligand to dissociate is present throughout all complex species.
<table>
<thead>
<tr>
<th>Mass-to-Charge (m/z) of Complex Precursors</th>
</tr>
</thead>
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<tr>
<td>C1P</td>
</tr>
<tr>
<td>404.9296*</td>
</tr>
<tr>
<td>550.6268</td>
</tr>
<tr>
<td>659.2889</td>
</tr>
<tr>
<td>439.91</td>
</tr>
</tbody>
</table>

Table 4. Selected Mass-to-Charge ratios for all complex precursors in positive ion mode. The last row indicates the expected mass-to-charge of each protonated species. *most prominent peak.

In the mass spectra for C3P and C4P, the most prominent peaks correspond to tolyl- and methoxy-terpyridine, respectively, where the molecular masses of each are 323.14 and 339.14 amu respectively. In addition to the dissociation of a chloro ligand, the terpyridine ligands of complexes C3P and C4P were observed to dissociate too. Because the UV/Vis spectra of the complex precursors suggested that the terpyridine was coordinated to the ruthenium metal center and not just unreacted terpyridine, the decision was made that the complex precursors synthesized were pure enough to be used in the subsequent reaction to form Cx.

V. Synthesis of Cx

Separating the two stereoisomers of our complex has proved a very difficult challenge. According to previous projects under Dr. Acquaye, the isomers can be separated through filtration as one isomer precipitates out during the reaction and the other remains soluble. However, the precipitate isomer was not observed to be soluble in common solvents like methanol, ethanol, acetonitrile, or water. Therefore, no spectral characterization studies were
performed. Therefore, soluble isomer was the representative compound for our DNA studies, however the specific conformation was never determined.

The problems with achieving purity mostly lied within the fact that our desired product could only be obtained in the filtrate. Earlier methods required one to rotary evaporate the mixture to complete dryness and wash with cold water and cold ethanol to retain most of our product, hoping that the unreacted materials would wash away. Nonetheless, the precipitate isomer still lingered and this was mostly observed in spectroscopic studies, as the precipitate would float about in the cuvettes. This incited another response, one that would separate the two isomers from each other and from the impurities.

Later methods, such as the one described in the synthesis of C2, was developed in response to the presence of even more possible impurities--C2 is the only compound whose terpyridine ligand had to be synthesized. The presence of more impurities was evident in the filtrate. Column Chromatography had to be employed to best separate the soluble isomer from other soluble impurities.

LC-MS analysis of our complexes before purification by column chromatography yielded spectra that were dominated by contaminants, however our product is still present in some samples. Despite the efforts to purify our desired products, the mass spectra did not resolve the problems with impurities. Table 5 shows all noticeable peaks for each complex.
### Mass-to-Charge (m/z) of chloro-Complexes

<table>
<thead>
<tr>
<th></th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
</tr>
</thead>
<tbody>
<tr>
<td>376.1221</td>
<td>360.0783*</td>
<td>374.0950</td>
<td>550.6282</td>
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</tr>
<tr>
<td>541.0055*</td>
<td>502.0362</td>
<td>657.1214</td>
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<tr>
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<tr>
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<td>799.1506</td>
<td>815.1461</td>
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<td></td>
<td></td>
<td></td>
<td>971.2071</td>
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<tr>
<td>709.11</td>
<td>785.14</td>
<td>799.15</td>
<td>815.15</td>
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</tbody>
</table>

**Table 5.** Selected Mass-to-Charge ratios for all complex precursors in positive ion mode. The last row indicates the expected mass-to-charge of each inherently cationic species. *most prominent peak.*

The presence of impurities is also translated in cyclic voltammetry in Figure 17, showing our best cyclic voltammogram across all complexes. There are two pairs of lumps that aren’t resolved which may be attributed to the two isomers within the solution. All other cyclic voltammograms did not have peaks of much height. An additional cyclic voltammogram is shown for reference.
Figure 17. Cyclic Voltammogram for a synthesis of aC3. This particular experiment produced the best result, meaning that the pairs of peaks were most defined.

Figure 18. Another rather “unsuccessful” cyclic voltammogram. This experiment was run with aC1.
To confirm that a reaction has occurred between CxP and L1, the UV/Vis spectra of CxP and Cx were compared. Figure 16 illustrates the differences in absorbance among each C1 form. As a result of the reaction, C1P would have to lose two chloro ligands, so we should expect a decrease in intensity for one peak. For the C1P spectrum, the peak at around 425 nm is relatively large compared to the terpyridine absorbance. However, when we look at the C1 spectrum, this peak decreases in intensity, again, relative to the terpyridine absorbance. The presence and intensity of that 425 nm peak is attributed to the amount of chloro ligands present on the complex. This trend is also evident in other overlay spectras. The absorbance at around 495 nm in C1P appears to shift to about 530 nm, and while this change could be attributed to the coordination of L1 to the ruthenium center, it could also be attributed to solvent conditions. Complexes dissolved in methanol were observed to produce a 530 nm peak, whereas complexes dissolved in dichloromethane produce a 495 nm peak—an instance of solvatochromism.

Across all Cx spectra, C2-4 has a distinctly sharp absorbance at around 495 nm, while this peak is absent in C1. This is illustrated in Figure 19. Because the structures of C2-4 all have a phenyl group attached to the terpyridine, this 495 nm peak is attributed to the presence of the fourth ring structure.
Figure 19. *The UV/Vis spectra for all chloro complexes, Cx.*

**VI. Synthesis of aCx**

To confirm that the chloro-form Cx has been converted into the aquo-form aCx, the UV/Vis spectra of Cx and aCx were compared. Figure 16 illustrates the differences in absorbances among each C1 form. As a result of the reaction, C1 would have to lose its last chloro ligand and acquire an aquo ligand. According to ligand field theory, aquo- is classified as a weak field ligand and chloro- is classified to be a weak, but stronger than the former, field ligand. We would expect that the chloro ligand splits the $t_{2g}$ and $e_g$ orbitals much more than the
aquo ligand. Therefore, higher energy light should be absorbed by Cx to excite the electrons into the $e_s$ orbital, whereas lower energy light should be absorbed by aCx.

We can observe this phenomenon by examining the C1 and aC1 spectra. aC1 uniquely absorbs at around 740 nm--due to the aquo ligand--and the intensity of the 425 nm peak decreases. This affirms the suggestion that the 425 nm peak is indeed due to the chloro ligand.

Upon LC-MS analysis of the aquo complexes, results obtained were rather similar to the chloro complexes: dissociation of the labile ligand (Cl or H$_2$O) and a variety of impurities/artifacts. Despite that the found m/z values for both the chloro and aquo forms were virtually the same, the aquo complex was considered to be truly different from the chloro complex based on the UV/Vis spectral data.

**DNA Studies:**

There are multiple ways a complex can bind to DNA and so multiple experiments must be conducted to assess the type of binding that occurs. Particular types of binding can be suggested or even expected based on the shape of the complex, hence why intercalative ligands are often planar with fused rings. To assess our particular complexes, experiments must be used in tandem to suggest and verify a particular binding mode since no complex-DNA crystals were able to be formed and analyzed via single crystal X-ray diffraction. These such methods are discussed below.
VII. Absorption Titration

Figure 20 below shows one absorption titration experiment for C4. The arrow indicates the change in absorbance as the concentration of CT-DNA in the cuvette increases. The hypochromatic shift indicates that the complex’s intercalation with DNA is energetically favorable. Because the entire spectrum was observed to be decreasing in absorbance, it is suggested that no phosphodiester backbone cleavage was involved in DNA-complex interaction. Cleavage of the backbone would cause the release of intercalators, so no net decrease in absorbance would be observed. This hypothesis can be tested with DNA cleavage via gel electrophoresis.

![Figure 20](image)

**Figure 20.** An absorption titration experiment with C4. Black arrows indicate the direction of change in absorbance as the concentration of DNA in the cell increases.

The intrinsic binding constant, $K_b$, can be determined in Equation 1. $\varepsilon_a$, $\varepsilon_b$, $\varepsilon_f$ are the molar extinction coefficients for the apparent, bound, and free metal complex respectively. Figure 21 shows the plot made to determine the intrinsic binding constant, $K_b$. 

44
\[
\frac{[\text{DNA}]}{(\varepsilon_a - \varepsilon_i)} = \frac{[\text{DNA}]}{(\varepsilon_b - \varepsilon_i)} + \frac{1}{K_b(\varepsilon_b - \varepsilon_i)}
\]  \hspace{1cm} (1)

**Figure 21.** An example of a successful absorption titration experiment plot for C4. The intrinsic binding constant, \(K_b\), is determined by the ratio of the slope to intercept. Average \(K_b\) (C4): \(5.37 \times 10^5 \text{ M}^{-1}\)

Table 6 displays the binding constants that could be resolved from the plot. The absence in intrinsic binding constants in Table 6 was derived from the erratic changes in absorbance as the concentration of CT-DNA increased within the reaction vessel. In most cases, the absorbance of the complex at a particular wavelength decreased upon the first few additions of DNA and then drastically increased upon subsequent additions. Other cases featured nonlinear changes in absorbance with respect to change in DNA concentration. A plot of just one of these “erratic” experiments are shown below in Figure 22.
<table>
<thead>
<tr>
<th>C1</th>
<th>aC1</th>
<th>C2</th>
<th>aC2</th>
<th>C3</th>
<th>aC3*</th>
<th>C4</th>
<th>aC4*</th>
</tr>
</thead>
<tbody>
<tr>
<td>n/a</td>
<td>2.66</td>
<td>n/a</td>
<td>tbd</td>
<td>4.15</td>
<td>2.59</td>
<td>5.37</td>
<td>0.538</td>
</tr>
</tbody>
</table>

Table 6. A summary of all absorption titration data. “n/a” indicates that the data plot was too erratic and no linear relationship between change in absorbance and [CT-DNA] was present.

*only one successful experiment, of three, with \( R^2 > 0.75 \).

**Figure 22.** An example of an erratic absorption titration plot. This particular case featured a decrease in absorbance followed by a steady increase in absorbance.

Failed experiments such as that shown in C1 were attributed to a few causes. One probable cause is the degradation of the complex by phosphate buffer. Although up to only 100 \( \mu L \) of phosphate buffer was added to the cuvette (of a total of 3 mL solution), that amount of phosphate buffer could be enough to degrade a quite significant amount detectable on the
spectrophotometer. Another cause may be from the presence of the precipitate isomer in the cuvette. The precipitate isomer may have induced turbidity unto the cuvette solution, increasing the overall absorbance. Shaking the cuvette to mix the addition of DNA would also “refresh” the turbidity.

All erratic experiments displayed hyperchromicity preceded by hypochromicity. These results may be explained by something analogous to thermodynamic versus kinetic products in organic chemistry. Perhaps intercalation is the initial interaction, but as the cuvette contents are incubated for a sufficient amount of time, the complex will begin to kink or covalently bind to the CT-DNA and release the intercalating complexes. These formerly intercalating complex molecules would then kink or covalently bind to more CT-DNA substrates. However, the more probable effect is the formation of covalent bonds because intercalation is indicated by competitive binding. All complexes are observed to have some intercalative properties which would not be present if the CT-DNA kinked and base pairs unaligned.

Absorption titration alone cannot determine the binding mode of our complex and several other methods must be employed. Although the intrinsic binding constant of our complex can be determined to quantify the binding strength of our complex, another method, competitive binding, will be used to qualitatively verify our complex’s binding strength.

VIII. Competitive Binding

Since our complexes had intrinsic binding constants within the magnitude of $10^5$ M$^{-1}$, we would expect that our complex is strong enough to compete with ethidium bromide. As shown in figure 23, aC4 successfully displaces ethidium bromide from the CT-DNA substrate.
Figure 23. A competitive binding with ethidium bromide experiment with C4. The black arrow indicates the decrease in intensity as the concentration of complex within the cuvette increases. The “EB” label indicates the fluorescence of the sample containing only ethidium bromide. “+DNA” label indicates the fluorescence of the sample containing only DNA and ethidium bromide.

The stern-volmer constant, $K_{sv}$, can be calculated with Equation 2 below, where $I_o$ is the “initial” intensity of the ethidium bromide bound to DNA without any complex, $I$ is the intensity of each varying concentration of complex in solution, and $[Q]$ is the concentration of the quencher i.e. the complex.

$$\frac{I_o}{I} = K_{sv}[Q]+1$$  \hspace{1cm} (2)

By plotting the concentration of complex in the cuvette versus the ratio of the initial intensity to the intensity of each sample, a linear relation can be obtained whose slope is equal to the stern volmer constant, $K_{sv}$ (M$^{-1}$). Figure 24 shows the resulting plot for an experiment for
aC4. The Stern-Volmer constant is a qualitative measure for the intercalative binding properties of the prospective complex. As stated in the introduction, if a non-linear plot is obtained, then the complex may be degrading to produce individual intercalators. A summary of all stern-volmer constants are organized in Table 7.

**Figure 24. A plot of C4 competitive binding data.** $K_{sv} = 2.92 \times 10^4 \text{ M}^{-1}$

<table>
<thead>
<tr>
<th></th>
<th>C1</th>
<th>aC1</th>
<th>C2</th>
<th>aC2</th>
<th>C3</th>
<th>aC3</th>
<th>C4</th>
<th>aC4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average $K_{sv}$ ($10^4 \text{ M}^{-1}$)</td>
<td>5.46</td>
<td>3.53</td>
<td>3.28</td>
<td>3.88</td>
<td>6.14</td>
<td>2.86</td>
<td>3.50</td>
<td>3.22</td>
</tr>
<tr>
<td>SD ($\pm 10^4 \text{ M}^{-1}$)</td>
<td>0.545</td>
<td>0.681</td>
<td>0.636</td>
<td>0.0732</td>
<td>0.0730</td>
<td>0.300</td>
<td>1.53</td>
<td>1.02</td>
</tr>
</tbody>
</table>

**Table 7. A summary of all stern-volmer constants determined by competitive binding studies with ethidium bromide.**

Judging from the values presented in Table 7, all aquo-forms are less potent intercalators than their respective chloro-forms; this trend slightly agrees with that of the absorption titration experiments. According to the *in vitro* mechanism between *cis*-platin and DNA, *cis*-platin replaces its chloro ligands with aquo ligands which serve as suitable leaving groups for forming
a bond with individual nucleobases. It is hypothesized that the aquo ligands of the presented aquo-complexes follow a similar mechanism, with some of the introduced complex population serving as intercalators and the remainder forming covalent bonds.

The $K_{sv}$ constant for C3 is greater than C1 which can be explained by the larger hydrophobic surface area of the tolyl terpyridine ligand. The larger the hydrophobic surface area, the more favorable of an interaction intercalation will be, since the environment between two base pairs is hydrophobic. This is the reason why C4 produces the smallest constant--the methoxy group disturbs the hydrophobicity of the ligand.

The $K_{sv}$ constants for all aquo-forms were observed to be less than their chloro-form counterparts. Tolyl terpyridine is more electron donating than terpyridine, so more electron density is donated into the ruthenium metal center for aC3 than aC1. Because electrons are being donated into ruthenium, we can assume that the bonds coordinating L1 and the aquo ligand are weaker. Therefore, the aquo ligand is likely to dissociate from the complex and the ruthenium complex cation ([Ru(L1)(tpty)]$^+$) will form a covalent bond to the DNA bases, much like the mechanism involving cis-platin, instead of intercalating with DNA.

With absorption titration, it was hypothesized that complexes eventually forfeit intercalation for a covalent binding mode. However, that is not observed in competitive binding. This can be explained by the way the experiments were run. For absorption titration, solutions were not remade and DNA was continually added to the same solution. This allowed the complexes to “pick-and-choose” the ultimate binding mode. With competitive binding, solutions were remade so complexes would only intercalate before the switching of binding modes.
The decrease in fluorescence could also be due to phosphodiester bond cleavage and subsequent release of ethidium bromide from the intercalative mode. In order to firmly determine whether intercalation is responsible for the decrease in fluorescence, thermal denaturation studies and DNA cleavage will be performed.

IX. Thermal Denaturation

Thermal denaturation was attempted with a Hitachi U-2900 Double-beam UV-Visible Spectrophotometer accompanied by a Lauda Eco E4G Heating Thermostat. There was no “communication” between the two, meaning that the temperature could not be monitored/altered with the spectrophotometer controls. Due to constraints on available instruments and machines, the method for thermal denaturation of CT-DNA in the presence and absence of ruthenium complex was performed in a rather crude manner. Thus, many problems were faced when trying to optimize this reaction.

a. Accurate $T_{\text{cuvette}}$ Measurements. Two methods were developed to record the temperature.

Method 1 measured the temperature of the tubes that carried the heated water from the Lauda water bath to the spectrophotometer. At the end of the experiment, the temperature of the cell was recorded with a thermometer and was found to be significantly lower than the temperature of the tubes. In order to get a more accurate depiction of the temperature, Method 2 directly measured the temperature of the water in the cuvette versus time (calibration phase)--at which the temperature of the water bath was increased whenever the temperature of the cuvette reached equilibrium. In the actual experiments, the temperature was altered in accordance to the calibration phase timeline, e.g. if equilibrium was reached
after 15 minutes increasing from 60-63 °C during the calibration phase, then at least 15 minutes were allowed to pass while the sample and reference cuvette reached equilibrium. The maximum temperature of the water bath was 97 °C and the maximum temperature of the phosphate buffer in the sample cuvette used in our study was 79.4 °C.

b. Precipitation of Complex. In order to only monitor the absorbance of DNA, complex was added to both the sample and reference cuvette. The complex was observed to precipitate out of solution once it was injected into the reference cuvette (containing only phosphate buffer) which lead to much fluctuation in the absorbance during the experimental phase. Our complexes are not soluble in the phosphate buffer, so this mistake was an oversight. To remedy this, a 1:1 v/v DMF:buffer solution was in both our sample and reference cuvettes which our complex would not precipitate out of. Additionally, the boiling point of DMF is much higher than water, unlike common organic solvents typically used to dissolve our complex.

c. Temperature Measurement of DMF:buffer Solutions. The specific heat capacity of DMF is higher than water, so another calibration phase had to be conducted for this new solvent mixture, however, the maximum $T_{\text{cell}}$ obtained was 72.6 °C, too low for the thermal denaturation curve to be remotely conceived. The solutions of each cell could not be substantially filled with DMF or any other solvent without the risk of dampening our maximum $T_{\text{cell}}$ or evaporating organic solvents (and concentrating our solution). However, samples that contained DNA and phosphate buffer only were allowed to fully dissolve the complex, suggesting there is a chemical interaction between the DNA and complex.
d. **Variation of Absorbance Mid-Experiment.** The absorbance of DNA (which is the absorbance of interest) came out at around 0.250 during the experimental phase. When complex is added to the solution, the absorbance spikes up to about 1.000 and the absorbance occasionally fluctuates by ± 0.004. To eliminate the absorbance spike and fluctuations, complex was added to the reference cuvette. Since the complex isn’t soluble in the phosphate buffer and DMF could not be added to the reference cell, the spike and fluctuations were tolerated.

e. **Incomplete thermal denaturation curve.** Thermal denaturation profiles typically extend to 95 °C and the melting temperature of the DNA or DNA-complex form could be estimated from the profile. Because our actual maximum $T_{\text{cell}}$ was only 79.4 °C, the melting temperature of the DNA or DNA-complex form could not be determined. Therefore, only qualitative analysis of the effects that a complex induces on the melting temperature of DNA could be performed and quantitative analyses were forfeited.

![Graph of thermal denaturation profiles and differential melting curves](image)

**Figure 25.** Thermal denaturation profiles (left) and differential melting curve (right) for DNA (a) and DNA-copper complex form (b). The copper complex intercalator was observed to increase the melting temperature of DNA by about 8 °C. The melting temperatures can also be calculated with differential equations.

Figure 25 above displays the thermal denaturation profile (left) and differential melting curves (right) for DNA with and without the presence of a copper complex intercalator.
In the thermal denaturation profiles, one can estimate the melting temperature of the DNA at the midpoint of the profile’s sigmoidal curve. From these values, one can determine by how many degrees Celsius does an intercalator increase the melting temperature of DNA. The change in melting temperature can also be estimated by examining the temperatures at which the absorbance begins to increase. Because our thermal denaturation profiles were never fully conceived, i.e. only a maximum temperature of 79.4 °C could be reached, I had to rely on the temperature at which the absorbance began to increase.

Despite all the complications, data was obtained for complexes which is summarized in Table 8. Judging from the temperature at which the absorbance begins to increase, or $T_0$ values, we can see that all complexes do intercalate with DNA, causing an increase in $T_0$ as a complex is added to the solution. C3, aC3, and C4 induce the most intercalation, followed by aC4, C1, and aC1. The capability for C3, aC3, and C4 to induce the most intercalation can be explained by the superiorly large hydrophobic surface area of the terpyridine ligand.

**Figure 26.** Raw data for the thermal denaturation profile of DNA in the presence of C3. $T_0$ for this experiment was taken at around 2400 s, which correlated to 72.9 °C. A sigmoidal curve is almost conceivable.
Table 8. A summary of data for experimental phase thermal denaturation studies of DNA and DNA in the presence of an added complex. $T_0$ represents the temperature at which the absorbance begins to increase from an initial $A_0$ and $A_f$ represents the absorbance of the complex at 79.4 °C. $T_f$ represents the final temperature of the cuvette directly measured with a digital thermometer once all other values were recorded. It is expected that the insertion of the thermometer probe would affect the absorbance after insertion.

The thermal denaturation experiment for C2 or aC2 were not performed yet because I am convinced the aquo-form was never synthesized, as denoted by the UV/Vis spectrum and fairly equal stern-volmer constant (compared to the chloro form, C2). Considering that the trend for C3/C4 species to intercalate more than C1 species is present in both thermal denaturation and competitive binding, it is likely that the binding modes occurring for our complexes are predominantly adduct formation and intercalation. With DNA cleavage, we can determine if phosphodiester cleavage in the presence of the complex still occurs.
X. DNA Cleavage

Figure 27. An altered image of agarose gel electrophoretic mobility pattern of plasmid DNA with the presented complexes of varying concentrations. Lane 1 is the molecular weight marker, lane 2 is the positive blank, and lanes 3-20 are samples with the complexes. An adjusted image to help visualize the band shifts is shown in Figure 28.

Agarose gel electrophoresis is a standard method used to study the separation of particles based on the particles’ size and charge. Small molecules are expected to move more quickly through the gel as a voltage is applied to the system. Supercoiled plasmid DNA, for example pUC18 -- the plasmid used in this experiment, is used to study a molecule’s ability to cleave phosphodiester bonds. Upon cleavage of the phosphodiester bonds, the more mobile supercoiled form (Form 1) should unwind into a relaxed, unwound, and less mobile form (Form 2). The amount of each form can be visualized with ultraviolet light if the agarose gel is equipped with ethidium bromide, as ethidium bromide intercalates with DNA and emits light upon excitation. It is expected that with increasing concentrations
of a cleaving complex with a fixed concentration of supercoiled plasmid DNA, more Form 2 should be present.

According to the experiment shown in Figure 27 (which is an altered form of the raw data, shown in Figure 28, to better visualize the band shifts), there is no noticeable conversion from form 1 to form 2 across any of our complexes. In the positive blank (lane 2), we can see the two forms. This is due to concentrations of the plasmid being too low inside of the wells. This was unexpected because the concentration of the plasmid DNA in each well was equal in both our experiment and the experiment done by Kozlyuk\textsuperscript{[39]}. Evidently, the experiment performed by Kozlyuk produced fairly distinct bands across all wells. If the complexes were to have no interaction with DNA, then we would see the same pattern in all other lanes, as the concentration of pUC18 was constant across all samples. However, we do not see this pattern in nearly all lanes. This is likely due to the complex intercalating with the DNA in place of ethidium bromide, and therefore the two forms cannot be visualized with UV light. In lanes 15 and 18, we see the most plasmid DNA -- these lanes also have the \textit{least} amount of complex present. The amount of each form is better visualized in Figure 27, which is a greatly modified image of Figure 28. As the concentration of the complex increases, the plasmid DNA is no longer visualized, indicating that either (1) the plasmid DNA has been denatured or cleaved into random sequences only a few nucleotides long and so migrate much faster, or (2) more of the complex is displacing ethidium bromide.

Lanes 15 and 18 also feature aquo-forms, aC3 and aC4, which, according to the other DNA experiments, were not observed to display as much intercalation with DNA as
their chloro counterparts. This could explain why lanes 15 and 18 display the most visualized plasmid -- the aquo forms are preferring adduct formation. Lanes 15 and 18 also do not display more of Form 2 than the positive blank, suggesting that there is no phosphodiester bond cleavage occurring.

It is possible that DNA cleavage by gel electrophoresis is not a suitable experiment for the presented complexes because the complexes are known to be good intercalators. Thus, the DNA forms cannot be visualized with UV light and ethidium bromide. It is risky and expensive to attempt this experiment again with higher concentrations of plasmid DNA because it is uncertain whether too much intercalation will occur for us to visualize the two forms.

Figure 28. The raw image of Figure 27.
CONCLUSIONS

Ruthenium complexes containing modified terpyridine ligands and a chloroquine analog were able to effectively bind to DNA and produce moderate binding constants. In tandem with all DNA reactivity studies intercalation is a definite binding mode for the complexes in question. It is also suggested that adduct formation also occurs for some complexes, especially the aquo forms. Aquo forms produced $K_{sv}$ constants less than their chloro forms, indicating intercalation occurs less in the former. In order to design an anticancer drug that intercalates as the primary form of DNA interaction (and ultimately induces apoptosis), aquo ligands should be absent in the final structure. Because the binding constants produced vary depending on the terpyridine modification, it is expected that terpyridine is the ligand that mostly participates in DNA binding, whereas the chloroquine analog may be an accessory ligand that stabilizes the DNA binding interaction. DNA cleavage indicates that our complexes may not be able to induce phosphodiester DNA backbone cleavage. However, this evaluation should remain inconclusive until further experimentation, as there is no hard evidence of the absence of DNA cleavage.
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